

Exhibit C

Special Issue Article

Development of a laboratory model to assess the removal of biofilm from interproximal spaces by powered tooth brushing

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ABSTRACT: ***Purpose:*** To develop an interproximal laboratory model to compare the potential effectiveness of powered brushing to remove biofilm plaque from interproximal spaces beyond the reach of bristles. ***Materials and Methods:*** *Streptococcus mutans* biofilms were first grown on glass microscope slides in a drip-flow reactor. The slides were removed and positioned in the interproximal model. Each slide was exposed to 15 seconds powered brushing with either the Sonicare Elite or the Braun Oral-B 3D Excel. The thickness of the biofilm was measured with confocal microscopy at various distances from the bristle tips. ***Results:*** The Sonicare Elite reduced the thickness of biofilm by 57% at a distance of 0-5 mm from the bristle tips, 53% at 5-10 mm and 43% at 10-15 mm, relative to biofilm in areas unexposed to brushing. All reductions in thickness were statistically significant ($P < 0.01$). The Braun Oral-B 3D Excel reduced the biofilm thickness by 16%, 13%, and 19% at the same distances respectively, but the thickness reductions were not statistically significant from those in the unexposed areas ($P > 0.1$). (*Am J Dent* 2002;15:12B-17B).

CLINICAL SIGNIFICANCE: The development of a model to assess the effectiveness of powered brushing to remove oral biofilms from interproximal spaces through fluid shear and bubble generation will help evaluate the development and potential ability of powered brushing strategies to control the development of oral biofilms.

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Introduction

Dental plaque biofilms are a complex community of microorganisms that grow on the hard and soft tissues of the oral cavity.^{1,2} Routine removal of dental plaque is critical in maintaining oral health, since the uninterrupted growth of plaque biofilms allows the formation of protective niches within which a succession of microorganisms that are the causative agents for caries,^{3,4} gingivitis and periodontal disease⁵ can proliferate.

The effects of periodontal disease include reversible gingival inflammation and irreversible destruction of periodontal tissues including the gingiva, periodontal ligament, and alveolar bone.⁶

Dental plaque is most commonly removed through routine tooth brushing and flossing.⁷ For manual toothbrushes the mechanical removal of dental plaque is achieved primarily through direct contact of toothbrush bristles and the scouring action of bristles across tooth and gum surfaces. However, recently a variety of powered toothbrushes have been developed to improve the efficiency of plaque removal using increased bristle velocity, brush stroke frequency, and various bristle patterns and motions. The Braun Oral-B 3D Excel[®] uses a rotary brush head motion in combination with pulsations along the bristle axis. The Sonicare Elite[®] toothbrush generates direct mechanical brushing and fluid motion by oscillating the bristles up and down from the gingival level to the occlusal surface (Fig. 1).

This study developed an *in vitro* interproximal (IP) model which could be used to evaluate the potential of the mechanical forces generated by powered toothbrushes to reduce the thickness of biofilm beyond the reach of bristles in

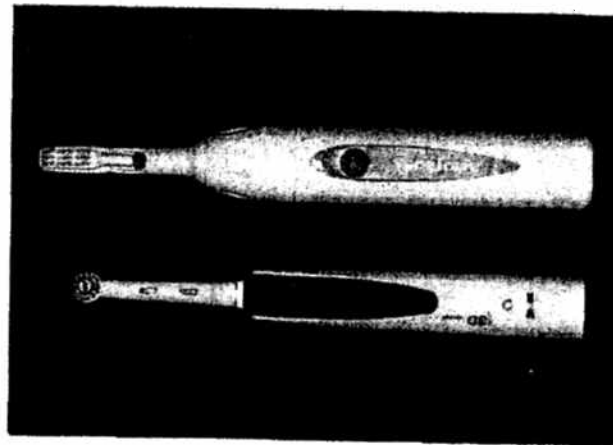


Fig. 1. The Sonicare Elite (top) and the Braun Oral-B 3D Excel (bottom).

the interproximal spaces of the teeth both quantitatively and qualitatively. The interproximal model was designed to simulate the exposure of the interproximal plaque between mandibular molar teeth to powered brushing from the buccal surface. Biofilms of *Streptococcus mutans*, an early colonizer of hard tooth surfaces,^{8,9} were grown on glass microscope slides in a drip-flow reactor, a simple and inexpensive system used to grow biofilms.¹⁰⁻¹² The colonized slides were then positioned in the interproximal model and exposed for 15 seconds to either the Sonicare Elite or the Braun Oral-B 3D Excel toothbrushes. The biofilm thickness at distances of 0-5, 5-10, and 10-15 mm from the bristles was measured by confocal microscopy and statistically compared with the thickness of biofilm in unexposed areas to estimate percent reduction by brushing.

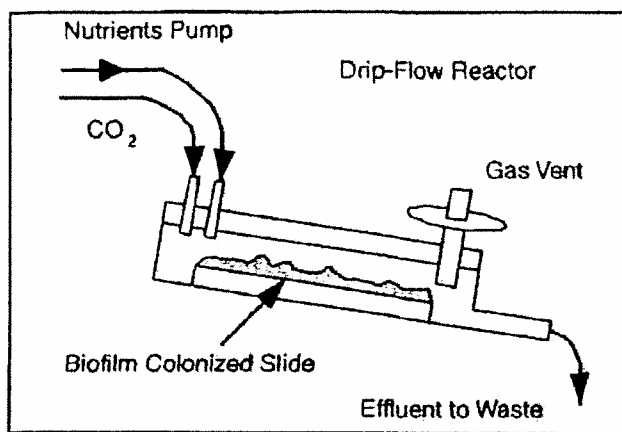


Fig. 2. Schematic of the drip-flow reactor system used to grow the *S. mutans* biofilms. The nutrients were dripped onto the glass slide and flow was gravity driven to the effluent waste line. A CO₂ headspace was maintained in the flow cell.

Materials and Methods

GROWTH OF BIOFILMS

Inoculum culture preparation - Thawed frozen stock culture (100 µl) of *S. mutans* UA159 was added to 100 ml Brain Heart Infusion broth supplemented with 2% sucrose. Cultures were incubated at 35°C overnight in an anaerobic chamber with CO₂ gas generating cartridges. Viable cell density of this culture was determined by serially diluting and drop plating onto solid Brain Heart Infusion agar + 2% sucrose. The plates were incubated overnight in an anaerobic chamber with CO₂ gas generating cartridges.

Drip-flow reactor preparation and sterilization - Biofilms were grown on four glass microscope slides each positioned in a channel of a four channel drip-flow reactor (Fig. 2).¹⁰ Nutrient feed and effluent lines were connected to the reactor with silicon tubing. The entire setup was autoclave sterilized for 20 minutes at 121°C.

Biofilm growth - Approximately 15 ml of Brain Heart Infusion broth + 2% sucrose was added to each of four chambers of a sterile drip-flow reactor. Three ml of an overnight culture of *S. mutans* UA159 was added to each chamber and the channel lids sealed by tightening with Nylon screws. The drip-flow reactor was incubated at 37°C in the presence of CO₂. The reactor was incubated without flow in a level position during for an initial 3-hour period to allow the *S. mutans* cells to attach to the glass slide. After the attachment phase, the drip-flow reactor was placed onto a block with a 10° incline and 1/10 Brain Heart Infusion broth + 2% sucrose media was pumped into each chamber at approximately 0.5 ml/minute for an additional 48 hours (Fig. 2).

EXPOSURE OF BIOFILM TO THE SONICARE ELITE AND THE BRAUN ORAL-B 3D EXCEL

Interproximal model - The interproximal model consisted of two acrylic chambers separated by a series of three aluminum posts representing mandibular molar teeth (Fig 3). A biofilm colonized glass microscope slide could be positioned adjacent to the first tooth post by vertically sliding it into place along two lateral guide grooves. The distance representing the interproximal space between the slide and the aluminum tooth post was 1 mm. The depth of the first tooth post was approximately 8.5 mm (7.4 edge-9.9 center mm). A seal was made be-

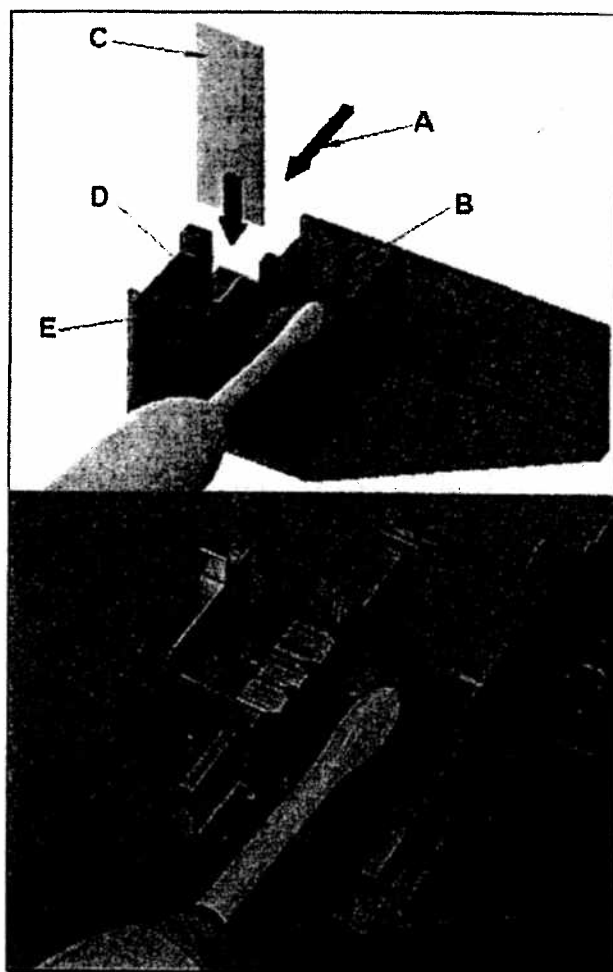


Fig. 3. Solid model drawing and photograph of the interproximal model, the biofilm colonized glass slide and the orientation of the Sonicare Elite toothbrush inserted in the interproximal space between the first tooth post. A) Real time visualization with zoom lens from this side. B) Frontal chamber. C) Microscope slide colonized with biofilm. D) Rear chamber. E) Teeth posts.

tween the glass slide and the acrylic body with a silicone "O" ring. The slide was held in place by two alligator clips. The chambers were filled with 30 ml of water so that the depth of the water was approximately 10 mm. During brushing the bristles were only partially submerged to simulate saliva coverage in the oral cavity. Both toothbrushes were positioned so that the bristles in the front portion of the toothbrush head were aligned with the interproximal space between the slide and the first artificial tooth and that the furthest extending bristle tips just made contact with the edge of the slide (0 mm bristle distance). According to manufacturer's recommendation, the Braun Oral-B 3D Excel was angled 90° with respect to the artificial dentition and the Sonicare Elite was maintained at a 45° angle.

Powered brushing in the interproximal model - A biofilm colonized slide was removed from the drip-flow reactor and placed inside a sterile Petri dish for transport. The slide was kept hydrated with sterile Ringers buffering solution. The slide was placed in the interproximal model, and then the model was filled to a depth of approximately 10 mm with sterile Ringers buffer solution. Final adjustments were made to position the

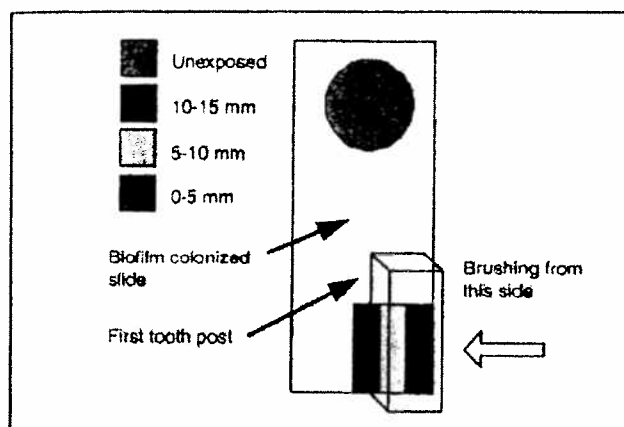


Fig. 4. Schematic showing location of measured biofilm thickness after exposure to 15 seconds powered brushing. Five thickness measurements were taken from the green unexposed area. Three thickness measurements were taken in each of the three other areas at increasing distance from the edge of the slide.

brush head and then the brush was activated for 15 seconds. The slides were removed from the chamber then stained using the Molecular Probes BacLight LIVE/DEAD kit[®] and incubated for 1 hour at 4°C. Live cells in the biofilm stained green and dead cells stained red. The brush and interproximal model were set up prior to the removal of each slide to minimize the time between removal from the growth chamber and brushing.

Biofilm structure and thickness measurements using confocal microscopy - A total of six independent drip-flow reactor growth cycles were used to generate a total of 24 biofilm colonized slides; of these, six were discarded because of visible sloughing of the biofilm or slide breakage during sampling. Two slides from each reactor were exposed to powered brushing from the Braun Oral-B 3D Excel and two slides were exposed to the Sonicare Elite so that in all, nine slides were used for each brush. After brushing and staining, each slide was examined using a Leica TCS-NT confocal microscope.⁴ The thickness of the biofilm was measured in four locations on the slide (Fig. 4). Three thickness measurements were made in each of three locations with increasing distance from the bristle tips. Three were taken 0-5 mm from the edge of the slide located closest to the bristles, three at a distance of 5-10 mm, and three at a distance of 10-15 mm (Fig. 4).

In addition, five thickness measurements were made in an unexposed area at the other end of the slide to serve as an internal control. The percent biofilm thickness reduction in each of the exposed interproximal areas relative to the unexposed area for each slide was calculated using the equation:

$$\% \text{ Biofilm Thickness Reduction} = \left(\frac{C-B}{C} \right) \cdot 100 \quad [1]$$

C = Unexposed biofilm thickness;
B = Exposed biofilm thickness; and
i = each exposed region

Statistical analysis - ANOVA between the thicknesses of biofilm in the exposed areas was compared to the thickness in the unexposed area using Minitab[®] (version 13.3). Differences between means were considered significant for $P < 0.01$. The mean values for biofilm thickness did not follow a normal distribution so a log transformation was performed to calculate the Standard Error and perform ANOVA to determine statistical significance between (1) brush types and (2) exposed



Fig. 5. Flow visualization by tracking the motion of bubbles coming through the interproximal space between the first tooth post and an uncolonized microscope slide positioned in the interproximal chamber. A) Movement of two air bubbles (white and yellow circles) over 1 frame interval (1/60 s). The grid lines were hand drawn (approx. 2.5 mm spacing) on the microscope slide to assist tracking. The red-dashed line indicates the distant edge of the first tooth post, the water level with brush activated is indicated by the blue line. B) The displacement of bubbles over successive video frames were found by subtracting each image from the previous image so bubbles that had moved from a location appeared dark (a) and those that had appeared were light (b). Bubble a-b was traveling at a velocity of 0.14 m/s and the bubble track at 'c' gave a velocity of 0.28 m/s.

regions. The ANOVA also calculated Repeatability Standard Deviation (RSD), which measures the repeatability between experiments and individual biofilms. Additionally, univariate ANOVA was performed on the grouped data from all slides to determine if there was a statistically significant difference between the unexposed biofilm thickness and the exposed biofilm thickness for each brush type in each exposed region.

Flow visualization during powered brushing - The movement of fluid during powered brushing was captured with a Sony power HAD 3CCD[®] color video camera using a 50 mm lens with a x20 zoom. Images were captured on both VHS (60 frames/second) and on a Scion VG-5 PCI[®] framestore board (20 fps) using Scion Image[®] software. Individual bubbles moving through the interproximal space were tracked by Scion Image and image subtraction showed how the position of a bubble changed over successive frames (Fig. 5). The bubble velocity was calculated by measuring the distance traveled over the frame interval.

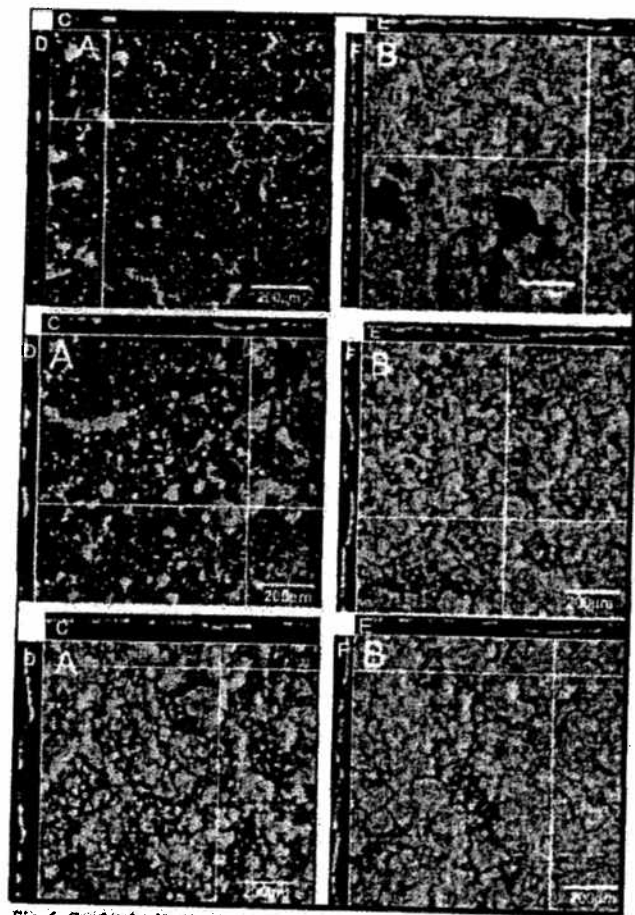


Fig. 6. Confocal micrographs showing the *S. mutans* biofilm in the area zones at distances of 0-5 mm (top), 5-10 mm (middle) and 10-15 mm (bottom) of the slide edge proximal to the bristles with the Sonicare Elite (A) and the Braun Oral-B 3D Excel (B) after 15 seconds powered brushing. Panels CD and EF are cross-sections in locations indicated by white lines. Scale bar = 200 μ m.

Results

Biofilm structure - After the 48-hour growth period, *S. mutans* formed an extensive biofilm on the glass slide. The biofilms were very heterogeneous with thickness that ranged from approximately 175 μ m to 1,400 μ m. Confocal microscopy images revealed that the individual cells in the biofilm were aggregated into cell clusters, which also varied in size from several cells to microcolonies of 200 μ m in diameter (Fig. 6). Secondary structures such as towers and channels were clearly visible throughout the biofilm. The thickness of the biofilm in the unexposed region of the slides used for brushing with the Sonicare Elite was 513 ± 258 μ m (mean \pm 1 S.D., $n = 45$) and 452 ± 277 μ m ($n = 45$) for the Braun Oral-B 3D Excel. There was no significant difference between the "unexposed" thickness in the two slide sets ($P = 0.196$).

Flow visualization - The Sonicare Elite produced large amounts of bubbles that were projected through the interproximal space between the first tooth post and the glass slide. Bubbles identified in the video sequences were traveling at a velocity of 0.24 ± 0.07 m/s ($n=5$), with the fastest measured at 0.38 m/s. Water traveling at a similar velocity in a parallel plate flow channel would generate a shear stress on the order of 0.9 Pa. However, many of the bubbles were moving too quickly to be discerned

Removal of biofilm from the interproximal space 15B

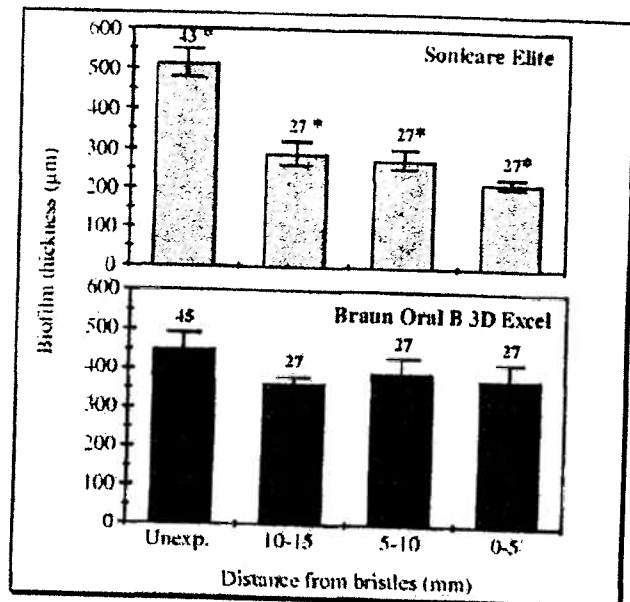


Fig. 7. Mean thickness of *S. mutans* biofilms in each of the exposed areas with decreasing distance from the slide edge in comparison to the unexposed area (unexp.). Bars are 1 standard error. Sample size n is shown above bar. Significant differences compared to the thickness in the unexposed area ($P < 0.01$) are indicated by "*".

in consecutive frames so that the bubble velocities reported here are likely to be an underestimate of the maximum velocity range. During some tests the visible removal of biofilm in patches of up to 1 cm^2 was observed while during other tests removal during brushing was less apparent but confirmed by the appearance of small pieces of detached biofilm in the interproximal water chambers (movies of this process can be viewed at www.erc.montana.edu/Res-Lib99-SW/Movies/default.htm). Bubbles were also generated by the Braun Oral-B 3D Excel but to a lesser extent. These bubbles were traveling at a velocity of 0.20 ± 0.03 m/s ($n=5$), which was not significantly different from the Sonicare Elite ($P = 0.36$, $n=10$). The fastest measured bubble was traveling at a velocity of 0.23 m/second. Fluid traveling at this velocity through the IP space would generate a corresponding shear stress of approximately 0.5 Pa. The removal of large pieces of biofilm was not seen during brushing for any of the nine slides exposed to the Braun Oral-B 3D Excel but detached clumps of biofilm were observed floating in the interproximal water chamber.

Influence of interproximal brushing on biofilm structure - Confocal examination of the biofilms after 15 seconds exposure to powered brushing with the Sonicare Elite showed that a substantial amount of biofilm, which in the unexposed area almost completely covered the slide, was removed, revealing large areas of the underlying glass slide (Fig. 6). The removal was greatest in the area closest to the bristles (0-5 mm) and differences between the biofilm in the unexposed area and the area at a distance of 10-15 mm from the bristles were more difficult to see. There was also biofilm removal by the Braun Oral-B 3D Excel, although not as extensive as the Sonicare Elite (Fig. 6).

There was no discernable difference in the distribution of red and green cells in the biofilm between the unexposed biofilm and the remaining biofilm exposed to either brush type, indicating that the main mode of action of the brushing on the reduction of viable biofilm cells was through physical removal.

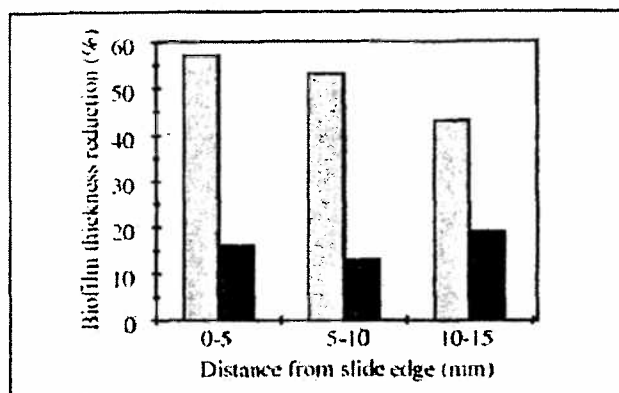


Fig. 8. Percent thickness reduction in *S. mutans* biofilm in each of the exposed areas with increasing distance from the bristle tips for the Sonicare Elite (yellow) and the Braun Oral-B 3D Excel (blue).

Influence of interproximal brushing on biofilm thickness - The quantification of detachment of the biofilm was estimated by measuring the thickness in the exposed and unexposed areas (Fig. 7). For both brushes the thickness of the biofilm was reduced in all of the measured areas in the vicinity of the interproximal space with respect to the thickness in the unexposed area. For the Sonicare Elite the thickness of the biofilm increased with distance away from the bristles. The greatest thickness reduction occurred in the area 0-5 mm from the bristles, which was $221 \pm 84 \mu\text{m}$ (compared with a thickness of $513 \pm 257 \mu\text{m}$ in the unexposed area). The Braun Oral-B 3D Excel did not show this trend and the biofilm thickness was similar in each of the three exposed regions. The thinnest biofilms occurred in the region 10-15 mm from the bristle tips with a thickness of $364 \pm 105 \mu\text{m}$ (compared with a thickness of $452 \pm 277 \mu\text{m}$ in the unexposed area). The differences in thickness in each of the three interproximal areas in relation to the unexposed area was statistically significant for the Sonicare Elite ($P < 0.001$) but not for the Braun Oral-B 3D Excel ($P > 0.109$).

The Sonicare Elite caused a thickness reduction of 57% in the area 0-5 mm from the bristle tips, 53% at 5-10 mm and 43% at 10-15 mm. This compared to reductions of 16%, 13% and 19% respectively for the Braun Oral-B 3D Excel (Fig. 8).

Statistical analysis - The variance between experiments performed on separate days was 0.006. The variance between individual biofilms was 0.041. Using these values, the Repeatability Standard Deviation (RSD) was 0.218 with 13% of the overall experimental variability attributed to experiments done on different days and 87% of the overall experimental variability attributed to individual biofilms. ANOVA showed that there was a significant difference between the biofilm thickness reduction obtained by the Sonicare Elite and that obtained by the Braun Oral-B 3D Excel ($P < 0.01$). There was a slight, but unexplained, difference in thickness reduction ($P = 0.045$) associated with biofilm grown on different days.

Discussion

Biofilm growth - The drip-flow reactor generated extensive *S. mutans* biofilms up to 1.5 mm thick after a 3-hour attachment period and 48-hour growth period. The drip-flow reactor provides an economical and easily operated system for growing biofilms. It is particularly well suited for use as an *in vitro* den-

tal model for the colonization of tooth surfaces since it provides a hard surface which is continually bathed in a thin film ($< 0.5 \text{ mm}$) of nutrients and contains a headspace where CO_2 can be easily introduced. The thickness and heterogeneity of the biofilms suggests that localized anoxic regions would develop in the biofilm¹³ demonstrating the potential of this system to cultivate more complex mixed community dental biofilms containing both aerobic and anaerobic organisms. The RSD showed that only 13% of the standard deviation was associated with day-to-day variability. This means that there was little error due to the differences in reactor setup or in human inaccuracies in biofilm thickness measurements. The majority of the variability, 87%, could, therefore, be attributed to the natural heterogeneity of the biofilm. However, the large variability in biofilm thickness, while possibly more representative of *in vivo* biofilms, is not necessarily ideal for comparative testing. Biofilm variability could possibly be reduced in future studies by decreasing the attachment and growth periods.

Interproximal model - The interproximal model integrated well with the drip-flow reactor because biofilms grown on the microscope slide could easily be transferred to the interproximal model and positioned in a manner approximating that of biofilm colonizing the interproximal spaces of the oral cavity. However, the thickness of the biofilm (measured in the unexposed areas of the slides used for both Sonicare Elite and Braun Oral-B 3D Excel tests) was highly variable ranging from $150 \mu\text{m}$ to $1413 \mu\text{m}$ with a mean of $482 \mu\text{m}$ ($n=88$). The standard deviation was 56% ($270 \mu\text{m}$) of the mean. The mean thickness of the biofilms used for the Sonicare Elite tests ($513 \mu\text{m}$) was 13% greater than the mean thickness of the biofilms used for the Braun Oral-B 3D Excel tests ($452 \mu\text{m}$), but this difference was not statistically significant. However, for comparative purposes it is desirable to reduce variability as much as possible, and although naturally grown biofilms often tend to be inherently heterogeneous,¹⁴ reduction of the attachment and growth phases may also reduce thickness and, therefore, variability. An advantage of the more complicated constant-depth film fermenter (CDFF), which has also been used to grow oral biofilms,¹⁵ is that thickness is tightly controlled by mechanically scraping off the top of the biofilm to a predetermined level. Additionally, for the present study glass slides were used so that the flow patterns could be monitored in real time by observation through the back of the slide, but for future studies hydroxyapatite coated slides could easily be used to provide a surface more similar to tooth enamel.

Flow visualization indicated that the Sonicare Elite produced more bubbles than the Braun Oral-B 3D Excel, but the camera frame capture rate of 60 fps was too slow to provide reliable quantification of numbers of bubbles generated and the velocities of the faster bubbles generated by the Sonicare Elite. However, image subtraction did provide an estimate of the flow velocity of the bubbles through the interproximal space. Although faster moving bubbles were seen with Sonicare Elite, the measured velocity was not statistically significant from the Braun Oral-B 3D Excel. However, the motion of bubbles through the simulated interproximal space was clearly visible and for future studies, capturing bubble motion with a high-speed camera may allow better tracking quantification and statistical comparison.

Reduction of biofilm thickness in the interproximal space by powered brushing - Confocal microscopy clearly showed the in-

fluence of powered brushing on the structure and thickness of biofilms. The trends reported here parallel those reported by Hope & Wilson¹⁵ using a different model of the interproximal space. In their model, biofilms were grown on hydroxyapatite disks in a constant-depth film fermenter. They also found the Sonicare Elite toothbrush to remove significantly more biofilm than the Braun Oral-B 3D toothbrush (32% vs. 9.5%, $P = 0.012$).

The effect of biofilm thickness reduction by the Sonicare Elite decreased with increasing distance away from the bristles as expected due to the dissipation of the turbulence by the fluid viscosity. However, even at 10-15 mm away from the bristle tips the thickness of the biofilm was still significantly reduced by 43%. This is relevant since interproximal spaces *in vivo* rarely exceed 10 mm. This trend complements previous studies in which viable cell counts were used to quantify the removal of biofilm by the original Sonicare Advanced toothbrush.^{16,17} Those studies report a greater than 60% reduction in *S. mutans* cells adhering to titanium at a distance of 4 mm from the bristle tips¹⁷ and nearly an 80% removal of human dental plaque at a 3 mm distance,¹⁶ both after 15 seconds of powered brushing. The Braun Oral-B 3D Excel also caused a reduction in biofilm thickness but only between a quarter to a half that caused by the Sonicare Elite. The increased biofilm reduction by the Sonicare Elite suggests that this brush produced greater mechanical forces in the interproximal space. This is consistent with a previous study¹⁸ reporting that a Sonicare powered brush produced significantly more force in the interproximal space than the Braun P35 in an *in vitro* model.

In addition to the fluid shear and normal forces created by the bristle motion, both brushes produced air bubbles due to a "beating" effect of the semi-submerged bristles. These bubbles were then forced through the interproximal space. The passage of air bubbles across a biofilm colonized substratum has been shown to remove up to 91% of cells on a conditioned glass surface.¹⁹ The same study also reported that detachment increased at lower bubble velocities because of the greater time allowed to develop the tri-phasic interface between the bubble, the fluid and the solid substratum. However, this detachment was from a sparse covering of recently attached cells in a monolayer, not the type of thick mature biofilm we used in our study. Additionally, it appears that the Sonicare Elite produces numerous bubbles over a wide range of sizes and traveling at a wide range of velocities. The role of bubbles on the detachment of mature biofilms could be investigated in a similar manner to that used by Gómez-Suárez *et al.*¹⁹ Biofilms grown in a parallel plate reactor could be exposed to increased fluid velocity (and therefore, shear) alone and also to the same shear but with introduced bubbles to find the relative contribution to bubble scouring and fluid shear on the detachment of oral-biofilms. However, the present study clearly demonstrated that the Sonicare Elite caused a significant reduction in biofilm thickness in an *in vitro* interproximal space at distances of over 10 mm from the bristles. It is expected that comparable mechanical forces applied *in vivo* would result in a similar effect. Further understanding on the role of mechanical forces on the detachment of biofilms from interproximal spaces and periodontal pockets will further enhance the design of powered toothbrushes to facilitate improved oral health care.

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